FORM PTO-1390 (REV. 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: P-23

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/889480

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CU00/00004	November 16, 2000	November 16, 1999

TITLE OF INVENTION: Antibodies and FV Fragment Recognizing Antigen IOR C2

APPLICANT(S) FOR DO/EO/US: Centro de Inmunologia Molecular

09/889480 Rec'd PCT/PTO 16 JUL 2001

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). /unsigned (signed Oath/Declaration to follow)
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. (to follow)
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. (to follow)
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. (to follow)
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Copy of the Deposit of the Microorganisms (to follow)
    Copy of PCT Request (PCT/RO/101) (to follow)
    Copy of International Search Report (PCT/ISA/210) (to follow)
    Copy of the International App. Publication (WO 99/2056) (to follow)

U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/889480 | INTERNATIONAL APPLICATION NO. PCT/CU00/00004 | P-23

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1,000.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☒ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	15 - 20 =	--	x **$18.00**	$ -0-
Independent claims	1 - 3 =	--	x **$80.00**	$ -0-
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ -0-

TOTAL OF ABOVE CALCULATIONS =	$1,130.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +	$ -0-
SUBTOTAL =	$1,130.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).	$ -0-
TOTAL NATIONAL FEE =	$1,130.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$ -0-
TOTAL FEES ENCLOSED =	$1,130.00
Amount to be refunded:	$
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a. ☒ A check in the amount of $ 1,130.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 10-0100 A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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Marvin Feldman,
NAME

25,197
REGISTRATION NUMBER

09-21-01

PCT

Serial or Patent No.: 09/889,480

P-23

Filed or Issued: July 16, 2001

Box 1.1 SEQ

For: Antibodies and FV Fragment Recognizing Antigen IOR C2

DECLARATION CLAIMING SMALL ENTITY STATUS

[37 CFR 1.9(f) and 1.27(c)]

SMALL BUSINESS CONCERN

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below

NAME OF CONCERN: CENTRO DE INMUNOLOGIA MOLECULAR

ADDRESS OF CONCERN: Calle 216 y 15, Atabey, Playa, C. Habana 12 100, Cuba

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled: Antibodies and FV Fragment Recognizing Antigen IOR C2 described in

- ☐ the specification filed herewith
- ☒ application serial no. 09/889,480, filed July 16, 2001
- ☐ patent no. , issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME: Agustin Lage Dávila ADDRESS: Calle 216 y 15, Atabey, Playa, C. Habana 12 100, Cuba	☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION
FULL NAME: ADDRESS:	☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION
FULL NAME: ADDRESS:	☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified declaration is directed.

NAME OF PERSON SIGNING	TITLE
Agustin Lage Dávila	Director of CIM
SIGNATURE	DATE
	August 1, 2001

RESIDENCE ADDRESS

Calle 216 y 15, Atabey, Playa, C Habana 12 100, Cuba

09/889480
JC18 Rec'd PCT/PTO 16 JUL 2001

ANTIBODIES AND FV FRAGMENT RECOGNIZING ANTIGEN IOR C2.

FIELD OF THE INVENTION

This invention is related to the field of the biotechnology and in particular with new recombinant antibodies obtained using genetic engineering technology, specifically with a chimeric antibody, a humanized antibody and a single chain Fv fragment obtained from murine IOR C5 antibody, which recognize epitopes expressed in ior C2 antigen which has been characterised as glycoprotein complex which is expressed in normal and malignant colorectal cells.

BACKGROUND OF THE INVENTION

They have been tested different forms of colorectal carcinoma treatment, however up to day the surgery it has been the only curative way. The surgery has allowed reaching higher percents of survival, when the detection of the tumour is in an early stage, but unfortunately the most cases are diagnosticated when the tumour has metastized.

In this moment, the strategy to increase survival includes the diagnosis, the therapeutic and epidemiology, in stages wherein it has not been produced the dissemination of the disease to external layers of the organs and the tumour is still surgically curable. In the way, the knowledge of epidemiological factors as well as the development of new therapeutically methods will help to increase the survival.

The use of monoclonal antibodies (Mabs) or their fragments, labelled with radioactive isotopes for the detection of cancer through immunogammagraphic methods, has been used in the last years. The Mabs have shown potential to be used as carriers of radioisotopes and to be targeted to the associated tumour antigens.

Some of the radiolabelled antibodies have been used to detect tumours associated with carcinoembrionary antigens (CEA). The antibodies against CEA, labelled with I-131 or I-125 are used to detect tumours producing CEA or associated with this marker (Patents US No. 3 663 684, US No. 3 867 363 y US No. 3 927 193). Also, Mabs can be labelled with Tc-99m to get molecules for "*in vivo*" diagnosis.

The development of the hybridoma antibody technique by Köhler and Milstein revolutionised the discipline of immunochemistry and provided a new family of reagents with potential applications in the research and clinical diagnosis of diseases (Köhler G; Milstein C. (1975) Nature 256, 495-497). These antibodies have not shown strong therapeutic efficacy, while it has become routine to produce mouse

monoclonal antibodies (mAbs) for use in basic research and clinical diagnosis, it has been difficult to use these for *"in vivo"* immunotherapy, because they have reduced half life in humans, poor recognition of mouse antibodies effector domains by the human immune system and also because foreign immunoglobulins can elicit an antiglobulin response (HAMA response) that may interfere with therapy.

The development of the genetic engineering has revolutionised the ability to genetically manipulate antibody genes and then to produce mAbs having decreased or eliminated antigenicity and enhanced desired effector functions, when these antibodies are used in the treatment or diagnosis of some pathologies. These manipulations have provided an alternative where a murine mAb can be converted to a predominantly human form with the same antigen binding properties (Morrison S. L; et al 1984, P.N.A.S. USA, 81,6851-6855).

Recently they have been developed some methods in order to humanise murine or rat antibodies and decrease xenogenic response against foreign proteins when they are used in humans.

One of the first intents to reduce antigenicity, has been by producing "chimeric" antibodies. In these molecules, the variable domains were inserted into human frameworks, in this way not only it can be reached the decrease of the immunogenicity but also the improvement of effector functions, because they are humans and therefore recognised by the immune system (Morrison S. L et al (1984) P.N.A.S, USA 81, 6851-6855). These chimeric molecules retain the recognition of the original antigen and its constant region is not immunogenic, although the immunogenicity against murine variable region is retained.

Other authors have attempted to build rodent antigens binding sites directly into human antibodies by transplanting only the antigen binding site, rather than the entire variable domain, from a murine antibody (Jones P.T et al (1986) Nature 321, 522-524, Verhoeyen M et al (1988) Science 239, 1534-1536). They have been developed some applications of this method by Rietchmann (Rietchmann L. et al (1988) Nature 332, 323-327; Quee C. et al (1989) P.N.A.S USA 86,10029-10033), however other authors have worked with reshaped antibodies, which included some murine residues in human FRs in order to recover the affinity for the original antigen (Tempest,P.R (1991) Biotechnology 9, 266-272).

Mateo et al. (US Patent Number US 5712120) described a procedure to reduce immunogenicity of the murine antibodies. In this procedure, the modifications are

restricted to the variable domains and specifically to the murine frameworks of the chimeric antibodies. Even more, these modifications are only carried out in the FRs regions with amphipatic helix structure, therefore are potential epitopes recognised by T cells. The method proposes to substitute the murine residues inside the amphipatic regions, by the amino acids in the same positions in the human immunoglobulines, of course the amino acids involved in the tridimentional structure of the binding site, it means Vernier's zone, canonical structures of the CDRs and the amino acid of the inter-phase between light and heavy chain are excluded.

The antibody modified by the method described by Mateo et al, retains the capacity of the recognition and binding to the antigen, that recognised the original antibody and it results less immunogenic because of this it is got an increase of the therapeutic efficacy. Through this procedure only few mutations are necessary to obtain modified antibodies that shown reduced immunogenicity compared with chimeric antibodies.

The IOR C5 murine monoclonal antibody (patent application WO 97/33916) is an IgG1 isotype, obtained from immunisation of Balb/c with SW1116 cells (colorectal adenocarcinoma), recognised an antigen expressed preferentially in the surface and cytoplasm of the malignant and normal colorectal cells. This antibody does not recognise neither CEA, Lewis a, Lewis b, asialylated Lewis, membranes of normal mononuclear cells antigens nor red globules (Vázquez A. M. et al, Hybridoma 11, pag. 245-256, 1992).

Western blotting studies using SW1116 membranes extract showed that this antibody recognized a glycoprotein complex which was denominated ior C2, with two molecular weight forms (145 and 190 Kda) (Vázquez A. M. et al, Year Immunol. Basel, Karger, vol. 7, pag. 137-145, 1993).

Also it is known from the state of the art that using genetic engineering techniques, recombinant fragments can be constructed from monoclonal antibodies. There are many reports validating the use of different antibody fragments in the *"in vivo"* diagnosis and the therapeutic of the diseases.

Ira Pastan et al. (EP 0796334 A1) describes the construction of single chain Fv fragments, using variables regions of antibodies that specifically recognised carbohydrates related with Lewis Y antigen. Using these fragments, he developed a method to detect cells bearing this antigen. Also, he gives evidences of the inhibitor effect of these fragments on cells bearing the antigen.

DISCLOSURE OF THE INVENTION

This invention is related to recombinant antibodies obtained using genetic engineering technology, specifically with a chimeric antibody, a humanised antibody and a single chain Fv fragment obtained from murine antibody IOR C5 antibody, produced by the hybridoma of the same name deposited in correspondence with the Budapest Treaty under accession number ECCC 97061101 with European Collection of Cell Cultures, on June 11, 1997. This antibody recognizes epitopes expressed in ior C2 antigen, which is a glycoprotein complex that it is expressed in normal and malignant colorectal cells.

DETAILED DESCRIPTION OF THE INVENTION

cDNA Synthesis and Gene Amplification of the variable region of murine C5.

Cytoplasmic RNA was extracted from about 10^6 hybridoma cells of the monoclonal antibody C5 (Vázquez A.M. et al. Year Immunol, Basel, Karger, vol 7, pag. 137-145, 1993). The method used to extract RNA was described by Faloro et al (Faloro,J.,Treisman,R.,and Kemen,R. (1989). Methods in Enzymology 65:718-749).

The cDNA synthesis reaction consisted of 5 ug RNA, obtained with 25 pmoles of the designed primers to hybridise in the beginning of the constant region of murine IgG1, and in the murine constant kappa region for the light chain, 2.5 mM each of deoxinucleotide (dNTPs), 50 mM Tris-Hcl pH 7.5, 75 mM KCl, 10 mM DTT, 8 mM $MgCl_2$ and 15 u of ribonuclease inhibitor (RNA guard, Pharmacia) in a total volume of 50 ul. Samples were heated at 70^0C, for 10 min and slowly cooled to 37^0C over a period of 30 min. Then, 100 units reverse transcriptase were added and the incubation at 42^0C continued for 1 hour.

The variable regions of light chain (VK) and heavy chain VH) were amplified using Polymerase Chain Reaction (PCR). Briefly, 5 µl cDNA of VH or VK were mixed with 25 pmoles of specific primers, 2.5 mM each of dNTP, 5 µl buffer 10X for the enzyme DNA polymerase and 1 unit of this enzyme. Samples were subjected to 25 thermal cycles at 94^0C, 30sec; 50^0C, 30sec; 72^0C, 1 min; and a last incubation for 5 min at 72 ^{0}C.

Cloning and Sequencing of Amplified cDNA.

The purified VH and VK cDNA were cloned into TA vector (TA Cloning kit. Promega, USA). Clones were sequenced by the dideoxy method using T7 DNA Pol (Pharmacia, Sweden).

Construction of chimeric genes.

The light and heavy chains variable regions were obtained by enzyme restrictions from TA vectors and cloned into expression vectors (Coloma M.J. et al., Journal of Immunological Methods, 152, 89-104, 1992).

The VH genes were cut from TA vector by EcoRV and NheI digestion, and cloned in PAH 4604 expression vector, an human constant IgG1 is included and histidinol resistance gene.

The resultant construction is C5VH-PAH4604. The VK genes were cut from TA EcoRV and SalI digestion and cloned in PAG4622. This vector contains resistance to the gpt and used kappa human constant region. The resultant construction is C5VK-PAG4622.

Chimeric antibody expression.

NSO cells were electroporated with 10 μg of C5VH-PAH4604 and 10 ug of C5VK-PAG4622 and linearized by digestion with PvuI. The DNAs were mixed together, ethanol precipitated and dissolved in 25 μl water. Approximately 10^7 NSO cells were grown to semiconfluency, harvested by centrifugation and resuspended in 0.5 ml DMEN together with the digested DNA in an electroporation cuvette. After 5 minutes on ice, the cells were given a pulse of 170 volts and 960 μF) and left in ice for a further 30 minutes. The cells were then put into 20 ml DMEN plus 10% foetal calf serum and allowed recovering for 48 hours. At this time the cells were distributed into a 96 -well plate and selective medium applied (DMEN, 10% foetal calf serum, 0,8 μg/ml mycophenolic acid, 250 μg/ml xanthine). Transfected clones were visible with the naked eyes 10 days later.

The presence of the human antibody in the medium of wells containing transfected clones was measured by ELISA. Microtiter plate wells were coated with goat anti-human (gamma chain specific, After washing with PBST (phosphate buffered saline containing 0.02% Tween 20, pH 7.5), 100 μl of culture medium from the wells containing transfectants was added to each microtitter well for 1 hour at 37^0C. The wells were washed with PBST and the conjugated goat anti- human Kappa, light chain specific were added and incubated at room temperature for one hour. The wells were then washed with PBST and substrate buffer containing dietanolamine added. After 30 minutes the absorbency at 405 nm was measured.

Construction of humanised IOR C5h by T epitopes humanisation.

Prediction of T epitopes.

The variable region sequences of IOR C5 were analysed using AMPHI program, which predicts segments of the sequences 7 or 11 amino acids in length with an amphipatic helix, which are related with T immunogenicity. Also it was used SOHHA program which predicts hydrophobic helix (Elliot et al. J. Immunol. 138: 2949-2952, (1987). These algorithms predict fragments related with T epitopes presentation in the light and heavy variable regions of the IOR C5.

Analysis of homology of variable regions.

The variable domains of IOR C5 are compared with those corresponding human variable domains, to identify the most homological human sequence with murine molecule. The human sequence databases used were reported in Gene Bank and EMBL, both of them available in Internet. The comparison was made by an automated-computerised method, PC-DOS HIBIO PROSIS 06-00, Hitachi.

Analysis for immunogenicity reduction.

The essence of this method lies in reducing the immunogenicity by humanisation of the possible T cell epitopes, with only few mutations in the FRs, specifically in the amphipatic helix, excluded the positions involved with the tridimentional structure of the binding site.

In this method it is compared VH and VK regions of the murine immunoglobuline, with the most homological human immunoglobuline sequence and it could be possible to identify the different residues between murine and human sequences, only inside the amphipatic regions, within the FRs zone (Kabat E.(1991) Sequences of proteins of immunological interest, Fifth Edition, National Institute of Health), only these murine residues will be mutated by those of the human sequence at the same position.

Those residues in the mouse framework responsible for the canonical structures or those involved in the Vernier zone can not be mutate, because they could have a significant effect on the tertiary structure and to affect the binding site. Additional information about the substitutions in the tertiary structure, could be obtain, doing a tridimensional molecular model of the variable regions.

Cloning and Expression of humanised IOR C5 antibody into NSO cells.

After doing PCR overlapping to get mutations and humanised VH and VK, the obtained genetic construction corresponding to IOR C5 by humanisation of T cell

epitopes, were cloned into expression vectors in a similar way as used for the expression of the chimeric antibody, yielding the following plasmids: C5Vkhu-PAG4622 and C5Vhhu-PAH4604. The transfection of these genes into NSO cells was done in exactly the same conditions that we previously described for the chimeric antibody.

Obtainment of single chain Fv fragment. Construction and expression of the scFv.

The strategy includes a first amplification using PCR, which modify VH and VL sequences, including the endonucleases restriction sites to clone in the expression vectors. The amplification used designed oligonucleotides on the exact sequence.

After amplifying, the variable regions are purified and digested with the corresponding restriction enzymes. The DNA fragments are purified and ligated to the expression vectors. Later, these genetic constructions are expressed in E. coli, following conventional methods.

In the extraction process of the protein from the producer cells, a rupture process by ultrasound is doing, and it is possible to separate the soluble and insoluble fractions combining SDS polyacrylamide electrophoresis gels, nitro-cellulose transfer and western blot.

Partial purification of the protein is carried out by a process which includes: (1) separation of the soluble and insoluble material by ultrasound and centrifugation, (2) Wash in low molarities of urea and solubilization in high concentrations of urea. From solubilized material, to purify the protein by affinity chromatography to metals ions. Later, the protein is renaturalised against buffer.

Examples

Example 1. Obtainment of the Chimeric monoclonal antibody.

The VH and VK cDNAs were obtained from RNA extracted from the hybridoma producing the monoclonal antibody IOR C5 using reverse transcriptase enzyme. The specific primers used were:

For VH:

5´AGGTCTAGAA(CT)CTCCACACACAGG(AG)(AG)CCAGTGGATAGAC 3´

For VK:

5´GCGTCTAGAACTGGATGGTGGGAAGATGG 3´

The ADNc of the chains VH and VK were amplified using polymerase chain reaction (PCR) with Taq polymerase enzyme, and using specific primers ECORV/NHEI restriction site for VH and ECORV/SALI for VK. The specific primers used were:

For VH:

Oligonucleotide 1:

5´GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT 3´

Oligonucleotide 2:

5´TGGGTCGAC(AT)GATGGGG(GC)TGTTGTGCTAGCTGAGGAGAC 3´

For VK:

Oligonucleotide 1:

5´GGGGATATCCACCATGAGG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)3´

Oligonucleotide 2:

5´AGCGTCGACTTACGTTT(TG)ATTTCCA(GA)CTT(GT)GTCCC3´

The PCR products were cloned in TA vector (TA cloning kit, Invitrogen). Twelve independent clones were sequenced by dideoxy method using T7 DNA Pol (Pharmacia). The VH and VK sequences have high relation with the sub-group 2 of Kabat.

Then, VH chain was digested ECORV/NHEI and VK, ECORV/SALI, and cloned in PAH4604 and PAG4622 for VH and VK respectively. These vectors were donated by Sherie Morrison (UCLA, California, USA), and they are used for the immunoglobulines expression in mammalian cells. The PAH 4604 vector has included human constant region IgG1 and the PAG 4622 has human Ck (Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction., M. Josefina Coloma et al, Journal of Inmunological Methods, 152 (1992), 89-104) The resultant constructions after clonig IOR C5 regions were VHC5-PAH4604 and VKC5-PAG4622.

NSO cells were electroporated with 10 ug of the chimeric vector C5VH-PAH4604 and 10 ug of C5VK-PAG4622 and linearized by digestion with PvuI. The DNAs were mixed together, ethanol precipitated and dissolved in 25 ul water. Approximately 10^7 NSO cells were grown to semi-confluence, harvested by centrifugation and resuspended in 0.5 ml DMEN together with the digested DNA in an electroporation cuvette. After 5 minutes on ice, the cells were given a pulse of 170 volts and 960 uF and left in ice for a further 30 minutes. The cells were then put into 20 ml DMEN plus 10% foetal calf serum and allowed to recover for 48 hours. At this time the cells were

distributed into a 96 -well plates and selective medium applied (DMEN, 10% foetal calf serum, 10mM histidinol). Transfected clones were visible with the naked eyes 10 days later.

The presence of chimeric antibody in the medium of wells containing transfected clones was measured by ELISA. Microtiter plate wells were coated with goat anti-human (gamma chain specific, Sara lab). After washing with PBST (phosphate buffered saline containing 0.02% Tween 20, pH 7.5), 20 ul of culture medium from the wells containing transfectants were added to each microtitter well for 1 hour at 37ºC. The wells were washed with PBST and alkaline phosphatase conjugated goat anti- human Kappa, light chain specific were added and incubated at room temperature for one hour. The wells were then washed with PBST and substrate buffer containing dietanolamine added. After 30 minutes the absorbance at 405 nm was measured.

Example 2. Obtainment of different versions of humanised antibody.

The VH and VK IOR C5 sequences were compared with a human sequences database, obtaining the most human homological sequence with the IOR C5.

Then the amphipatic regions or possible T cell epitopes, were determined in VH and VK regions.

For VH, mutations were introduced in positions 10 and 17, and the amino acids ASP and SER by GLY and THR respectively, were substituted. These mutations were done by PCR overlapping, using primers 1 and 2, 3 and 4 in a first PCR and the results of these PCR were overlapped in a second PCR, using 2 and 4 primers, whose sequences are the following: (Kamman, M., Laufs, J., Schell, J., Gronemborg, B. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). Nucleic Acids Research 17:5404,1989).

Primers for the mutations 10 and 17 of the heavy chain.

Primer 1:

5' GAGTCAGGACCTGGCCTGGTGAAACCTTCTCAGACACTTTCACTCACC 3'

Primer 2:

5´ TGGGTCGAC(AT)GATGGGG(GC)TGTTGTGCTAGCTGAAGAGAC 3´

Primer 3:

5' GGTGAGTGAAAGTGTCTGAGAAGGTTTCACCAGGCCAGGTCCTGACTC 3'

Primer 4:

5´ GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT 3´

After the former mutations were verified by sequencing, new mutations were introduced to this mutated DNA, the new mutations introduced in positions 43 and 44 were LYS and GLY, substituting ASN and LYS respectively. The overlapping procedure was done as the previous overlapping. The mutations were verified by sequencing, this new construction was called C5VHhu.

The primers described for these mutations were:

Primers for the mutations 43 and 44 in the heavy chain.

Primer 1:

5' CAGTTTCCAGGAAAAGGACTGGAATGGATG 3'

Primer 2:

5´ TGGGTCGAC(AT)GATGGGG(GC)TGTTGTGCTAGCTGAAGAGAC 3´

Primer 3:

5' CATCCATTCCAGTCCTTTTCCTGGAAACTG 3'

Primer 4:

5´ GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT 3´

For VK the mutations were done in positions 15, 45 y 63 substituting ILE, LYS and THR, by LEU, ARG y SER, respectively.

The mutations were introduced by overlapping PCR as describe previously. The sequences of the used primers are shown. The new genetic construction was named C5Vkhu.

Primers for the mutation 15 of the light chain.

Primer 1:

5' TTGTCGGTTACCCTTGGACAACCAGCC 3'

Primer 2:

5´ AGCGTCGACTTACGTTT(TG)ATTTCCA(GA)CTT(GT)GTCCC 3´

Primer 3:

5' GGCTGGTTGTCCAAGGGTAACCGACAA 3'

Primer 4:

5´ GGGGATATCCACCATGAGG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)CT(TG)GT

Primers for the mutation 45 of the light chain.

Primer 1:

5' GGCCAGTCTCCAAGGCGCCTAATCTAT 3'

Primer 2:

5´ AGCGTCGACTTACGTTT(TG)ATTTCCA(GA)CTT(GT)GTCCC 3´

Primer 3:

5' ATAGATTAGGCGCCTTGGAGACTGGCC 3'

Primer 4:

5´ GGGGATATCCACCATGAGG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)CT(TG)GT

Primers for the mutation 63 of the light chain.

Primer 1:

5' CCTGACAGATTCAGTGGCAGTGGATCA 3'

Primer 2:

5´ AGCGTCGACTTACGTTT(TG)ATTTCCA(GA)CTT(GT)GTCCC 3´

Primer 3:

5' TGATCCACTGCCACTGAATCTGTCAGG 3'

Primer 4:

5´ GGGGATATCCACCATGAGG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)CT(TG)GT

All the mutations were verified by sequence.

The humanised VK and VH were cloned into the vectors PAG4622 and PAH4604, the followings constructions were obtained, C5Vkhu-PAG4622 and C5VHhu-PAH4604.

The NSO cells were electroporated with 10 µg of the humanised C5VHhu-PAH4604 and 10 µg of the C5VKhu-PAG4622. These vectors were linearized with PVUI digestion.

The electroporation and detection of the clones expressing humanised antibody IOR C5h were identical to the previous described for the chimeric antibody.

Example 3. Construction of the single chain Fv fragment:

Construction of the scFv fragment (VH-linker-VL), from variable domains (VH y VL) of IORC5 mAb. Cloning into expression vector to express in E.Coli.

Procedure (a). Construction of the scFv.-

The strategy has a first round of amplification by PCR, modifying sequenced VH and VL regions, including restriction endonucleases sites to cloning into the expression vectors pPACIB.7plus and pPACIB.9plus. In the amplification, the oligonucleotides designed under the exact sequence are used.

Heavy Chain:

4066: EcoRV-FR1-VH

5´.GGGATATCTGAGGTGCAGCTTCAGGAGTCAGGA..3´

4255: EcoRV-FR4-VH

5´..CAGGATATCGCAGAGACAGTGACCAGAGTCCC..3´

Light Chain:

2938: Sal I-FR1-VL

5´.CGTCGACGATATCCAGATGAC(AC)CA(GA)ACT(AC)C..3´

2935: Apa I- FR4-VL

5´.ATGGGCCCTTT(TC)A(TG)(TC)TCCAGCTTGGT..*3´*

After amplifying the regions, were purified and digested VH (EcoRV) and VL (SalI-ApaI). The DNA fragments were purified and ligated with pPACIB.9plus and pPACIB.7plus, vectors, previously digested with restriction enzymes.

The plasmid pPACIB.7plus is modified to export to periplasm heterologous proteins whose genes are expressed in *E.coli.* This plasmid contains regulatory sequences to get the following functions: Promoter sequence (tryptophan), sequence for signal peptide (OMPA), sequence for linker peptide (Chaudhary et al., 1990) and a domain composed by 6 hystidines codified in matures protein's C-terminal to help in the purification of this protein (Gavilondo, J.V. et al. Proceedings of the IV Annual Conference on Antibody Engineering. IBC Conferences Inc. Coronado, CA. December 8-10, 1993).

The plasmid pPACIB.9plus (Figure 1) is modified to express in the cytoplasm heterologous proteins whose genes are expressed in *E.coli.* This plasmid contains regulatory sequences to get the following functions: Promoter sequence (tryptophan), 27aa fragment derived from IL-2h for getting efficient expression of the protein, and a domain of 6 hystidines codified in matures protein's C-terminal to help in the posterior purification of this protein (Gavilondo, J.V. et al. Proceedings of the IV Annual Conference on Antibody Engineering. IBC Conferences Inc. Coronado, CA. December 8-10, 1993).

The PCR reaction's product was used to transform the competent *E.coli* cells (strain MC1061), which were plated under solid selective medium and grown at 37°C. To select recombinant vectors, a bacterial colonies were inoculated in liquid medium and extracted plasmid DNA from this culture (Molecular Cloning, A Laboratory Manual, second edition,1989, Sambrook, Fritsch and Maniatis). The plasmid DNA was digested by EcoRV, SalI/ApaI, XhoI/ApaI according cloning step, after applying under agarose gel and visualised with UV light, the recombinant clones were select between the clones with digestion pattern of two bands, one of them corresponding to pPACIB.7 and 9plus (approx. 2.9kb), and the second to the expected domain

(approx. 320pb VH or VL y 720pb for the scFv). For VH domain the insertion orientation was checked by DNA sequencing.

Procedure (b). Expression of scFv in *E.coli*, obtained from variable domain genes of IOR C5 Mab.

Four strains of E. coli were transformed (TG1, coliB, W3110 y MM294), to study the cloned gene expression, using two recombinant plasmids selected in (a). Basically the recombinant bacteria were grown in liquid medium (LB) with ampicillin, overnight at 37°C. From these cultures, were inoculated fresh cultures containing ampicillin, and incubated by 3 hrs at 37°C. Then, the expression of the protein was induced, adding to the culture beta-indolacrylic acid (inductor of the tryptophan promoter). The analysis of the samples in SDS poliacryilamide gels at 12%, indicated that a protein of approximately of 28kDa is expressed under these conditions, in the periplasmatic fraction for the construction of pPACIB.7plus and a 30 kDa band for the recombinant clone in pPACIB.9plus, which is expressed in TG1 in between 6-11% of the total bacterial protein. It demonstrated through a Western blot (Molecular Cloning, A Laboratory Manual, second edition de 1989, by Sambrook, Fritsch and Maniatis) with an antisera obtained in rabbit against Fab fragment of IOR C5 Mab, and immuno-purified, that this protein corresponds to scFv of IOR C5.

Example 4. Obtention of the scFv from bacterial cultures, renaturalisation and recognition assays to antigen.

Procedure (a). Extraction and renaturalisation of the scFv of IOR C5 from recombinant clone in pPACIB.9plus.-

In the extraction process of the protein from the producer cells using rupture ultrasound process, that allowed to separate soluble and insoluble fractions, combining with SDS-polyacrilamide electrophoresis gels, transferred to nitro-cellulose and Western blot, evidenced that the protein remains in the insoluble bacterial fraction.

Under these circumstances the protein was partially purified in a process including the followings steps:

(1) separation of the soluble and insoluble material by ultrasound and centrifugation,

(2) wash in low molarities of urea (2 M) and

(3) solubilization to high molarities of Urea (6 M).

From the solubilized material, the protein is purified in affinity chromatography to metallic ions and renaturalised against buffer solution.

Procedure (b). Binding assay to tumour cells of the scFv-IORC5 fragment.

Cell lines:

The cells were obtained from Centro de Inmunología Molecular. SW948 adenocarcinome cell line was grown in L-15 medium supplemented with 10% bovine foetal serum at 37°C in 6 % CO_2. Raji cell line (Burkitt human limphome) and Hut 78 (T human cell line) were used as negative controls.

These cell lines were grown in RPMI 1629 supplemented with 10% bovine foetal serum at 37°C.

The cell suspensions were fixed to 10^6 cell/ml in PBS containing 1% albumin of bovine serum. 10 ul of cell suspension was added to each well. The slides were dried in the dusty free air during 3 hours and fixed in acetone-methanol (1:1) solution, 5 minutes, and hydrated in TBS by 10 minutes. Finally, the cells were processed, using immunocytochemistry assay.

The activity of scFv IORC5 fragment was determined using immunocytochemistry assay, trough immunoperoxidase technique. The cells were incubated during 2 hours at 37°C with single chain Fv IOR C5, followed by incubation with anti Fab serum and with an anti-mouse peroxidase conjugated (HRPO), each one for 30 minutes at room temperature. The localisation site of the peroxidase were visualised with solution which contains 5 mg of 3-3 diaminobencidine, 5 ml of TBS and 5 µl of H_2O_2, 30 %. Between incubations, the slides were washed with cold TBS.

After introducing in water, the slides were contrasted with Hematoxilline of Mayer and Canadian Balsam was added. Each experiment included positive and negative controls.

The immunocytochemistry studies revealed that this fragment is only positive to SW948 cell line, that showed a moderate labelled comparing with the complete Mab, demonstrated a specific recognition of the scFv IORc5 to this cell line. The label was associated to the membrane and cytoplasm compartment in the malignant colon cells.

Brief description of the Figures.

Figure 1: Shows the genetic construction of the plasmid pPACIB.9plus, which is a modified plasmid to express fusion proteins in the cytoplasm of *E.coli*. This plasmid contains regulatory sequences to get the following functions: Promoter sequence (tryptophan), 27aa fragment derived from IL-2h for getting efficient

expression of the protein and, a domain of 6 histidines codified in mature protein's C-terminal to be used during the purification of this protein.

SEQUENCE LISTING

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(Applicant only for United States of America)

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CLAIMS

1. Recombinant antibody and single chain Fv fragment derived from murine monoclonal antibody IOR C5 produced by the hybridoma deposited under number ECCC 97061101, wherein said recombinant antibody has the Complementary Determining Regions (CDRs) of the antibody IOR C5 and human constant regions for light and heavy chains.

2. Recombinant antibody according to claim 1 wherein the CDRs sequences of the light and heavy chains are the following:

HEAVY CHAIN

CDR1: S D Y N W H

CDR2: Y I S Y N G T T S Y N P S L K S

CDR3: N D E K A W F A Y

LIGHT CHAIN

CDR1: K S S Q S L L D S D G K T Y L N

CDR2: L V S K L D S

CDR3: W Q G T H F P H T

3. Recombinant antibody according to claims 1 and 2 which is a chimeric antibody derived from murine monoclonal antibody IOR C5 which contains the CDRs and framework regions (FRs) of the antibody IOR C5 and human constant regions of the light and heavy chains, wherein said framework amino acid sequences of the heavy and light chains are the following:

HEAVY CHAIN

FR1: D V Q L Q E S G P G L V K P S Q T L S L T C T V T G Y S I T

FR2: W I R Q F P G K G L E W M G

FR3: R I S I T R D T S K N Q F F L Q L N S V T T E D T A T Y Y C A R

FR4: W G Q G T L V T V S A

LIGHT CHAIN

FR1: D V V M T Q T P L T L S V T L G Q P A S I S C

FR2: W L L Q R P G Q S P R R L I Y

FR3: G V P D R F S G S G S G T D F A L K I R R V E A E D L G V Y Y C

FR4: F G G G T K L E I K R K S T L T G

4. Recombinant antibody according to claims 1 and 2 which is a humanised antibody derived from murine monoclonal antibody IOR C5 that contains point mutations in the framework regions of the heavy and light chains for reducing its immunogenicity.

5. Humanised antibody according to claim 4 which has in the framework regions of the heavy and light chains any of the following point mutations

HEAVY CHAIN:

Position 10 ASP por GLY

Position 17 SER por THR

Position 43 ASN por LYS

Position 44 LYS por GLY

LIGHT CHAIN:

Position 15 ILE por LEU

Position 45 LYS por ARG

Position 63 THR por SER

6. Single chain Fv fragment according to claim 1, comprising the following sequences of the frameworks and CDRs for the variable regions of the light and heavy chains:

HEAVY CHAIN

FR1: D V Q L Q E S G P G L V K P S Q T L S L T C T V T G Y S I T

FR2: W I R Q F P G K G L E W M G

FR3: R I S I T R D T S K N Q F F L Q L N S V T T E D T A T Y Y C A R

FR4: W G Q G T L V T V S A

CDR1: K S S Q S L L D S D G K T Y L N

CDR2: L V S K L D S

CDR3: W Q G T H F P H T

LIGHT CHAIN

FR1: D V V M T Q T P L T L S V T L G Q P A S I S C

FR2: W L L Q R P G Q S P R R L I Y

FR3: G V P D R F S G S G S G T D F A L K I R R V E A E D L G V Y Y C

FR4: F G G G T K L E I K R K S T L T G

CDR1: K S S Q S L L D S D G K T Y L N

CDR2: L V S K L D S

CDR3: W Q G T H F P H T

7. Cellular line expressing the recombinant antibody of any of claims 1 to 5.
8. Host cell which express the single chain Fv fragment of claims 1 and 6.
9. Pharmaceutical composition for treating recto and colon malignant tumours, metastasis thereof and recurrences, comprising the recombinant antibody of any of claims 1 to 5 and a suitable excipient.
10. Pharmaceutical composition for treating recto and colon malignant tumours, metastasis thereof and recurrences, comprising the single chain Fv fragment of claims 1 and 6 and a suitable excipient.
11. Pharmaceutical composition for localisation and identification *"in vivo"* of recto and colon malignant tumours, metastasis thereof and recurrences, comprising the recombinant antibody of any of claims 1 to 5.
12. Pharmaceutical composition for localisation and identification *"in vivo"* of recto and colon malignant tumours, metastasis thereof and recurrences, comprising the single chain Fv fragment of claims 1 and 6.
13. Pharmaceutical composition according claims 9 to 12 comprising also compounds for radiolabelling these antibodies o fragments, which are mixed to produce an aqueous administrable solution.
14. Pharmaceutical composition according claim 13 comprising tecneciun 99, rhenio 186, rhenio 188 or analogues as radiolabellers.
15. Diagnostic method to identify *"in vivo"* recto and colon malignant tumours, metastasis thereof and recurrences, comprising a physiologically acceptable composition which contains any of the antibodies of claims 1-5 or the fragment of claims 1 and 6, which previously have been labelled with Tc-99m or any analogue, and the monitoring of the biodistribution of this composition by immunogammagraphy methods.

ABSTRACT

The invention relates to the obtention of novel recombinant antibodies from murine antibody IOR C5 produced by the hybridoma deposited with the ECCC 97061101 according to the Budapest Treaty. Said recombinant antibodies were obtained using recombinant DNA technology and are characterized in that they recognize antigen ior C2. The recombinant antibodies are specifically chimeric antibody, humanized antibody, and single chain Fv fragment. The chimeric antibody contains the variable domains of the murine immunoglobuline and the constant regions of the human immunoglobuline. The humanized antibody contains the constant regions of human immunoglobuline and has been specifically modified in the murine frameworks regions (FRs) and within the latter, in those areas that may result in an antigenic site for cells T. The Fv fragment contains the variable domains of murine immunoglobuline. The invention also relates to the utilization of recombinant antibodies derived from murine antibody ior C5 in the diagnosis and therapy of colorectal tumors, the metastasis thereof and recurrences.

FIGURE 1

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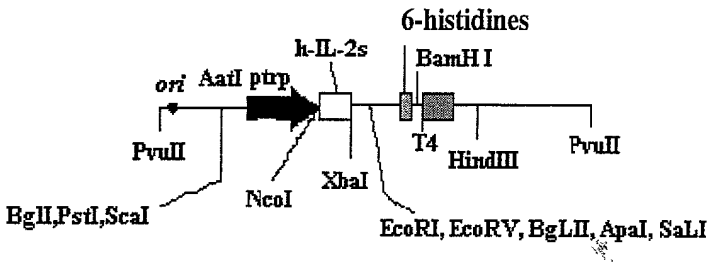

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Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Lys Ser Thr Leu Thr
1 5 10 15

Gly

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INVENTOR(S): MATEO DE ACOSTA DEL RIO, Cristina Maria, et al.

TITLE: Antibodies and Fv fragments recognizing antigen IOR-C2.

DOCKET NO:P-23

Assistant Commissioner of Patents
Washington, D.C. 20231

TRANSLATOR'S DECLARATION

Sir:

The undersigned, Josefa Lombardero, hereby declares: That I am a resident of CUBA residing at Agustina # 70, Vibora, Ciudad Habana,

That I am conversant in the Spanish and English languages and qualified to prepare an English translation from the corresponding Spanish language document.

That I have translated the attached English document from Spanish and that it is complete and adequate and it is a true and faithful translation of the original Spanish text, and

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and believe are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United State Code, and that such willful false statements may jeopardize the validity of the patent application.

Dated: 23 July, 2001

Translator's Signature

ENTERED PCT09

RAW SEQUENCE LISTING DATE: 03/22/2002
PATENT APPLICATION: US/09/889,480 TIME: 11:41:32

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  3 <110> APPLICANT: CENTRO DE INMUNOLOGIA MOLECULAR
  4       MATEO DE ACOSTA DEL RIO, Maria Cristina
  5       ROQUE NAVARRO, Lourdes Tatiana
  6       MORALES MORALES, Alejo
  7       PEREZ RODRIGUEZ, Rolando
  8       AYALA AVILA, Marta
  9       GAVILONDO COWLEY, Jorge Victor
 10       DUENAS PORTO, Marta
 11       BELL GARCIA, Hanssel
 12       RENGIFO CALZADO, Enrique
 13       IZNAGA ESCOBAR, Normando
 14       RAMOS ZUZARTE, Mayra
 16 <120> TITLE OF INVENTION: ANTIBODIES AND Fv FRAGMENT THAT RECOGNIZE Ior C2 ANTIGEN.
> 17 <130> FILE REFERENCE: CIM
> 18 <140> CURRENT APPLICATION NUMBER: US/09/889,480
> 18 <141> CURRENT FILING DATE: 2001-09-20
 18 <150> PRIOR APPLICATION NUMBER: PCT/CU00/00004
 19 <151> PRIOR FILING DATE: 2000-11-16
> 20 <160> NUMBER OF SEQ ID: 14
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49 <210> SEQ ID NO: 3

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RAW SEQUENCE LISTING DATE: 03/22/2002
PATENT APPLICATION: US/09/889,480 TIME: 11:41:32

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RAW SEQUENCE LISTING DATE: 03/22/2002
PATENT APPLICATION: US/09/889,480 TIME: 11:41:32

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VERIFICATION SUMMARY DATE: 03/22/2002
PATENT APPLICATION: US/09/889,480 TIME: 11:41:33

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UNITED STATES -- PATENT
DECLARATION FOR PATENT APPLICATION

Attorney's Docket No.: P 23

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint invent
(if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitl

the specification of which

(check one)

☐ is attached hereto.
☒ was filed on July 16, 2001 as
Application Serial No.: 09/889,480,
and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, amended by any amendment referred to above.

☐ I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Fede Regulations, §1.56(a).

☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate havi a filing date before that of the application on which priority is claimed:

☐ Prior Foreign Application(s):

Appln. No.	Country	Date Filed	Priority Claimed
196/99	CUBA	16/11/1999	☒ YES ☐ NO
PCT/CU00/00004	PCT	16/11/2000	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below an insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose informati which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available betwe the filing date of the prior application and the national or PCT international filing date of this application:

Appln. Serial No.	Filing Date	Status: Patented, Pending, Abandoned
		☐ Patented ☐ Pending ☐ Abandoned
		☐ Patented ☐ Pending ☐ Abandoned
		☐ Patented ☐ Pending ☐ Abandoned

POWER OF ATTORNEY

I hereby appoint the following attorney(s) and/or agent(s) to prosecute the application entitled
Antibodies and FV Fragment Recognizing Antigen IOR C2
and to transact all business in the Patent and Trademark Office connected therewith:

HENRY A. MARZULLO, JR., Reg. No. 20,910; MARVIN FELDMAN, Reg. No. 25,797
HOWARD N. ARONSON, Reg. No. 27,302; and
MYRON GREENSPAN, Reg. No. 25,680.

Address all telephone calls to *Myron Greenspan,* at telephone number (914) 723-4300, or to the attorney executing the last document. Address all correspondence to **LACKENBACH SIEGEL MARZULLO ARONSON & GREENSPAN, P.C.** at **Penthouse Suite, One Chase Road, Scarsdale, New York 10583 U.S.A.**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor Mateo de ACOSTA DEL RIO, Cristina María	Citizenship **Cuban**
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Date 23 July, 2001	Signature
Full Name of Second Joint Inventor Roque Navarro; Lourdes Tatiana	Citizenship **Cuban**
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State or Country Cuba	State or Country
Date 23 July, 2001	Signature
Full Name of Third Joint Inventor MORALES MORALES, Alejo	Citizenship **Cuban**
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State or Country Cuba	State or Country
Date 23 July, 2001	Signature

☒ **Additional inventors are being named on separately numbered sheets attached hereto.**

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State or Country Cuba	State or Country
Date 23 July, 2001	Signature

Full Name of Fifth Joint Inventor	Citizenship
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State or Country Cuba CUX	State or Country
Date 23 July, 2001	Signature

Full Name of Sixth Joint Inventor	Citizenship
GAVILONDO COWLEY, Jorge Víctor	Cuban
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City (Zip) Ciudad de la Habana	
State or Country Cuba CUX	
Date 23 July, 2001	Signature

Full Name of Seventh Joint Inventor	Citizenship
DUEÑAS PORTO, Marta	Cuban
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City (Zip) Ciudad de la Habana	City (Zip)
State or Country Cuba CUX	State or Country
Date 23 July, 2001	Signature

Full Name of Eighth Joint Inventor	Citizenship
BELL GARCÍA, Hanssel	cuban
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Calle 62 No. 906 Apto. 16 entre 9 y 11, Playa	
City (Zip) Ciudad de la Habana	City (Zip)
State or Country Cuba CUX	State or Country
Date 23 July, 2001	Signature

☒ **Additional inventors are being named on separately numbered sheets attached hereto.**

Full Name of ~~Sixth~~ ninth Joint Inventor RENGIFO CALZADO, Enrique	Citizenship **Cuban**
RESIDENCE Address – Street Calle 170 No. BCE2 Apto. 16, Playa	POST OFFICE Address – Street **(same as residence)**
City (Zip) Ciudad de la Habana	City (Zip)
State or Country Cuba CUX	State or Country
Date 23 July, 2001	Signature

Full Name of ~~Seventh~~ tenth Joint Inventor IZNAGA ESCOBAR, Normando	Citizenship **Cuban**
RESIDENCE Address – Street Ave. 31 No. 32005 entre 320 y 322, Eraga, La Lisa	POST OFFICE Address – Street **(same as residence)**
City (Zip) Ciudad de la Habana	City (Zip)
State or Country Cuba CUY	State or Country
Date 23 July, 2001	Signature

Full Name of ~~Eighth~~ eleventh Joint Inventor ~~RAMOS ZUZARTE, Mayra~~	Citizenship **Cuban**
RESIDENCE Address – Street Calle 184 No. BEE1, Apto. 12, entre 1ra y 5ta, Playa	POST OFFICE Address – Street **(same as residence)**
City (Zip) Ciudad de la Habana	
State or Country Cuba CUY	
Date 23 July, 2001	Signature

Full Name of Seventh Joint Inventor	Citizenship **Cuban**
RESIDENCE Address – Street	POST OFFICE Address – Street **(same as residence)**
City (Zip)	City (Zip)
State or Country	State or Country
Date	Signature

Full Name of Eighth Joint Inventor	Citizenship
RESIDENCE Address – Street	POST OFFICE Address – Street
City (Zip)	City (Zip)
State or Country	State or Country
Date	Signature

☒ **Additional inventors are being named on separately numbered sheets attached hereto.**